Induction of Potent Human Immunodeficiency Virus Type 1-Specific T-Cell-Restricted Immunity by Genetically Modified Dendritic Cells

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A novel technology combining replication- and integration-defective human immunodeficiency virus type 1 (HIV-1) vectors with genetically modified dendritic cells was developed in order to induce T-cell immunity. We introduced the vector into dendritic cells as a plasmid DNA using polyethylenimine as the gene delivery system, thereby circumventing the problem of obtaining viral vector expression in the absence of integration. Genetically modified dendritic cells (GMDC) presented viral epitopes efficiently, secreted interleukin 12, and primed both CD4$^+$ and CD8$^+$ HIV-specific T cells capable of producing gamma interferon and exerting potent HIV-1-specific cytotoxicity in vitro. In nonhuman primates, subcutaneously injected GMDC migrated into the draining lymph node at an unprecedentedly high rate and expressed the plasmid DNA. The animals presented a vigorous HIV-specific effector cytotoxic-T-lymphocyte (CTL) response as early as 3 weeks after a single immunization, which later developed into a memory CTL response. Interestingly, antibodies did not accompany these CTL responses, indicating that GMDC can induce a pure Th1 type of immune response. Successful induction of a broad and long-lasting HIV-specific cellular immunity is expected to control virus replication in infected individuals.

Cytotoxic T lymphocytes (CTL) are associated with the control of viremia in human immunodeficiency virus type 1 (HIV-1)-infected patients (21, 29) and simian immunodeficiency virus (SIV)-infected monkeys (9, 13, 41). However, currently available therapeutic approaches do not induce HIV-1-specific immunity. On the contrary, CD4$^+$- and CD8$^+$-mediated T-cell responses decline in time in patients treated with highly active antiretroviral therapies (14, 30, 31). The absence of HIV-1-specific cellular immunity contributes to treatment failures and to viral rebound after interruption of therapy. Encouraging results recently indicated that both HIV-1- and SIV-specific T-cell responses could be enhanced with early treatment of acute infection and that potent T-cell immunity was associated with immune control of virus after interruption of therapy (21, 23, 36). Although these results provided a rationale to explore immunotherapeutic approaches, the use of wild-type HIV-1 for autoimmunization raised safety concerns. Therefore, novel therapeutic vaccine approaches that are able to induce HIV-specific cellular responses are required to control virus replication. Here we describe a new technology to induce potent HIV-specific T-cell responses using genetically modified dendritic cells (GMDC).

MATERIALS AND METHODS

Construction of plasmid DNA. The replication- and integration-defective viral vector pLW/int was derived from a dualtropic primary HIV-1 isolate, laboratory worker (LW) (22). The mutant HIV-1 vector contains six stop codons and one deletion in the *pol* reading frame and one stop codon and one deletion in the second frame. First, the deletion was made. Second, multiple stop codons were introduced into the central *EcoRI-EcoRI* (nucleotides 4647 to 5742) fragment covering the integrase gene by primary PCR followed by overlap PCR using synthetic primers. The mutant fragment was cloned into a subclone with a deletion of the relevant fragment of the wild-type provirus. The authenticity of the final clone was checked by DNA sequencing.

Culture and HIV-1 infection of primary lymphocytes, macrophages, and DC. Peripheral blood lymphocytes and monocyte-derived macrophages were isolated from human and macaque peripheral blood as described earlier (6). Human and monkey dendritic cells (DC) were cultured for 7 days in complete culture medium (RPMI 1640 with 10% fetal calf serum) supplemented with 1,000 U of granulocyte-macrophage colony-stimulating factor and 700 U of interleukin 4 (IL-4) as described elsewhere (40). The wild-type virus, LW, and the integrase mutant (LW/int) viral vector were produced by transfection of 293 T cells with the corresponding plasmid DNA. Supernatants were collected 2 days after transfection and normalized for p24 before infection of primary human cells. Electron microscopic examination of the pLW- and pLW/int-transfected 293 T cells demonstrated comparable levels of viral particle production (data not shown).

Transduction of DC with plasmid DNA. DC (2×10^5/well) were plated in 150 µl of Optimem culture medium (Gibco) in a 96-well plate. Polyethylenimine (PEI) or PEI-mannose was used at an N/P ratio of 5 equivalents to complex about 2 µg of plasmid DNA (4). Cells were incubated for 4 h at 37°C. Half of the medium was replaced with fresh RPMI 1640 medium containing 10% fetal calf serum and cytokines (granulocyte-macrophage colony-stimulating factor and IL-4). Transduction of DC was routinely done in parallel wells. HIV-1 expression was monitored by a p24 antigen capture assay (Coulter).

Analysis of in vitro T-cell priming. DC (stimulator cells) were cocultured with autologous peripheral lymphocytes (responder cells) in a 1:10 ratio. Aliquots of this culture were analyzed after 3 days for gamma interferon (IFN-γ) production and after 7 days for CTL activity. For restimulation, an autologous B-lymphoblastoid cell line (B-LCL) primed with peptides or Zn-inactivated HIV$_{MN}$ or microvesicle (control supernatant obtained from the same cell line in the absence

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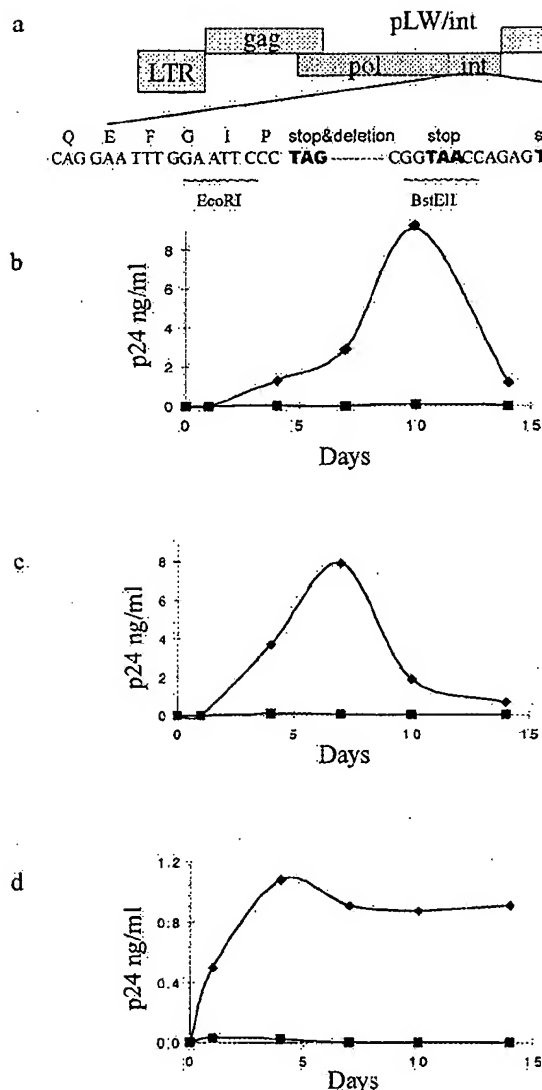† Present address: University of Muenster, 48149 Muenster, Germany.

FIG. 1. Characterization of the antigen. (a) Molecular clone encoding the integrase-defective HIV-1 (LW/int) vector. This plasmid can express full-length Tat, Rev, Nef, Vpr, Vpu, Vif, Gag, reverse transcriptase, and envelope proteins derived from the HIV-1 LW primary isolate (22) and a truncated integrase protein. LTR, long terminal repeat. (b through d) Infection of primary human lymphocytes (b), macrophages (c), and DC (d) with wild-type HIV-1, LW (◆), and the integrase-mutant retrovirus vector LW/int (564). In contrast to the parental wild-type virus (LW), the integrase-mutant virus was unable to induce productive infection in primary human cells.

of HIV$_{MN}$) was used. For flow cytometry, cells were incubated with 2 μg of brefeldin A per ml for 3 h and harvested for cytokine staining. Cells (5 × 10⁵) were washed twice with phosphate-buffered saline (PBS) containing 1% bovine serum albumin at room temperature and stained with PC5-CD3⁺ (UCHT1; Immunotech) and fluorescein isothiocyanate-CD8⁺ (B9.11; Immunotech) antibodies for 30 min on ice. Cells were washed twice with PBS and fixed in 0.5 ml of 4% paraformaldehyde solution for 15 min on ice. Cells were again washed twice with PBS and then permeabilized in 0.5 ml of 0.1% saponin solution for 15 min on ice, spun down, and resuspended in 40 μl of 0.1% saponin. These cells were stained with IFN-γ-phycoerythrin (45.15; Immunotech) antibody for 30 min on ice, washed twice with 1 ml of 0.1% saponin, and resuspended in 0.5 ml of PBS containing 1% bovine serum albumin for fluorescence-activated cell sorter analysis. All such analysis was controlled with isotype control staining.

In vivo localization of GMDC. Autologous monocyte-derived rhesus DC were transduced with DNA containing the neomycin phosphotransferase gene (neo). Ca. 500,000 were injected subcutaneously into the upper thigh of a rhesus macaque. One day later the draining lymph node was removed, fixed, and analyzed by in situ hybridization using a ³²P-labeled neo-specific antisense probe (and sense probe for control) and with immunohistochemical staining using p55 antibody (and isotype control) specific for lymph node DC (43).

GMDC vaccination of monkeys. Two colony-born pigtailed macaques (97280 and 96052) approximately 18 months of age were used in this study. Animals were treated and housed following current AALAC guidelines. All procedures were approved by the University of Washington Animal Care and Use Committee. Macaques were inoculated with ca. 500,000 autologous GMDC subcutaneously for localization studies. Vaccination was performed with 500,000 autologous GMDC. 250,000 GMDC injected into the saphenous vein and 250,000 GMDC injected subcutaneously in the upper thigh.

CTL assay. For human cells isolated from buffy coat, GMDC and control DC were cultured for 7 days with autologous T cells at a ratio of 1:10. T cells were collected and used as effectors in a CTL assay. Since buffy coat donors were not identified, we could not use autologous B-cell lines as target cells for the CTL assay. Instead, we used either freshly prepared autologous monocytes/macrophages or DC. Briefly, peripheral blood mononuclear cells (PBMC) depleted of T cells were plated in six-well plates in complete culture medium for 1.5 h. Nonadherent cells were then removed, and the remaining cells were incubated with fresh culture medium for an additional 7 days. After that time, all cells were collected and either left intact or incubated for 2 h with 10 μg of recombinant protein. Then the cells were pulsed with ⁵¹Cr (sodium chromate; Amersham) for 1 h. The cells were then washed three times and used as targets in a standard 4-h ⁵¹Cr release assay.

CTL analysis of macaque lymphocytes was performed essentially as described previously (15). We generated recombinant vaccinia virus-infected autologous B-LCL cells (obtained through herpesvirus papio transformation) as target cells. The B-LCL cells were pulsed with ⁵¹Cr prior to coculture, and ⁵¹Cr release from the cells was measured in the supernatant after 4 h. Responder cells were either autologous PBMC to measure effector CTL or autologous PBMC restimulated with autologous monocytes (obtained through adherence to plastic) infected with the appropriate recombinant vaccinia virus (obtained through the AIDS Research and Reference Reagent Program, National Institutes of Health).

RESULTS

We postulated that the use of naturally expressed and processed viral proteins might be advantageous for the induction of HIV-1-specific T-cell immunity, because an effective control of HIV-1 and SIV has been induced only by replication-competent (wild-type or attenuated) viruses (8, 21, 23, 36, 38). We wanted to overcome the safety problems associated with replication-competent viruses, so we decided to use a replication-defective HIV-1 vector as a source of antigens. Replication-defective retroviruses have been used as vectors for delivering therapeutic genes in experimental human gene therapy protocols and proved to be safe in HIV-1-infected patients but have not been shown to be effective in raising immune responses (27, 35). We mutated the integrase gene of HIV-1 because integrase-mutant retroviruses are not only replication defective but also unable to introduce permanent genetic modification in infected cells (28, 44). We began with a plasmid DNA containing an integrase-mutant HIV-1 vector (6) derived from a primary isolate that was able to infect both macrophages and T lymphocytes (22). The safety of the DNA was further in-
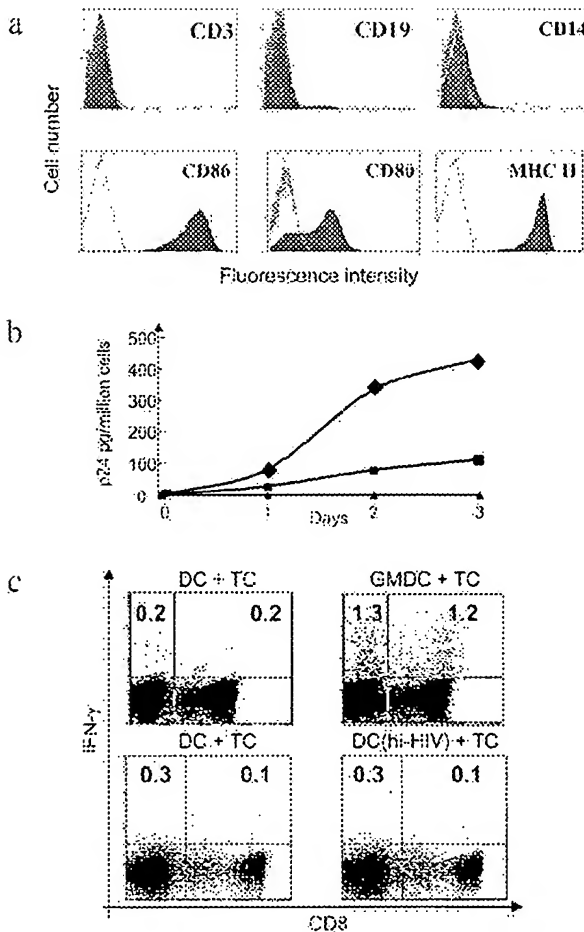
FIG. 2. HIV-1 vector expressing GMDC can prime naïve T cells in vitro. (a) Characterization of monocyte-derived DC by isotype control and antibody staining. (b) GMDC expressing the HIV-1 vector. DC were transduced with plasmid DNA containing wild-type HIV-1 (pLW) (♦), a plasmid carrying the integrase-defective mutant (pLW/int) (■), and a control plasmid encoding the green fluorescent protein (Clontech) (▲) using PEI-mediated gene delivery. (c) Priming of naïve T cells (TC) by DC, GMDC, and DC pulsed with hi-HIV-1. IFN-γ production was analyzed in T cells with a flow cytometer 3 days after priming.

ture and process antigens, express costimulatory molecules, secrete cytokines, and contact T cells to initiate immune responses (2). Movement of antigens to the lymphoid organs may be critical for the induction of T-cell immunity (19, 48). DC have the unique capacity to present antigens in the lymphoid organs and induce antigen-specific CTL activity by priming naïve CD4$^+$ and CD8$^+$ lymphocytes (3, 7, 18, 24).

Our challenge was to express the proteins encoded by the retrovirus vector in DC. To circumvent the problem of viral vector expression in the absence of integration, we introduced the HIV-1 vector into DC as plasmid DNA. We found that PEI, a nonviral gene delivery vehicle, can be used for this purpose. PEI is a versatile cationic polymer that condenses DNA (4). The PEI-DNA complex enters cells via endocytosis (20). PEI buffers endosomes, and the resulting osmotic swelling liberates the complex into the cytoplasm (4). PEI also facilitates the trafficking of DNA into the nucleus, thus aug-
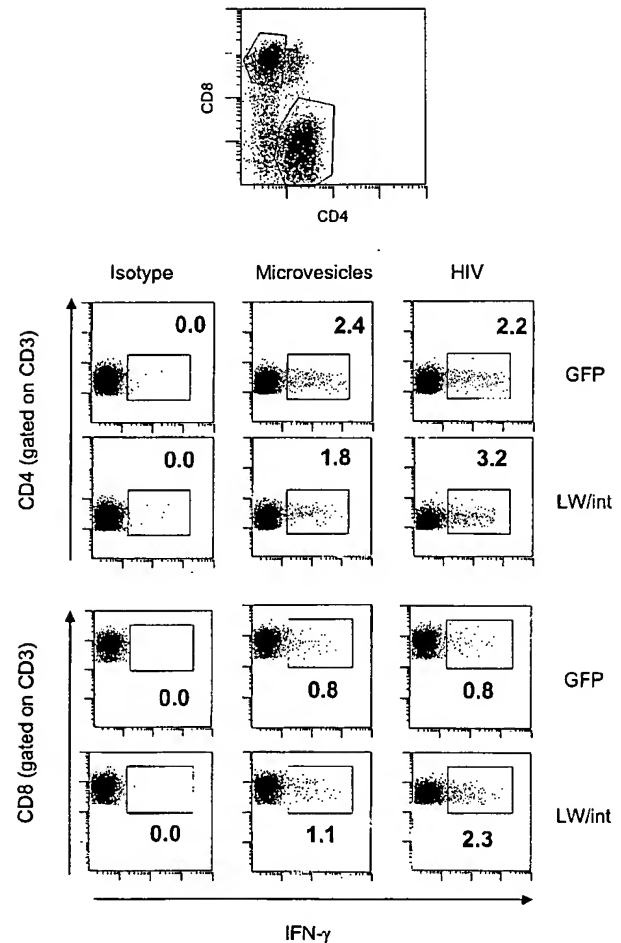

FIG. 3. Characterization of GMDC-primed T cells. Naïve T cells were primed with GMDC transduced with either LW/int or control pGFP plasmid DNA and cultured for 14 days. T cells were restimulated with autologous B-LCL cells primed with either Zn finger-inactivated HIV or microvesicle control (gift from Jeff Lifson, NCI, Frederick, Md.). (Top panel) Gating of CD4$^+$ and CD8$^+$ T cells on the CD3$^+$ population. Labels on the right and the top indicate antigens used for priming and restimulation, respectively.
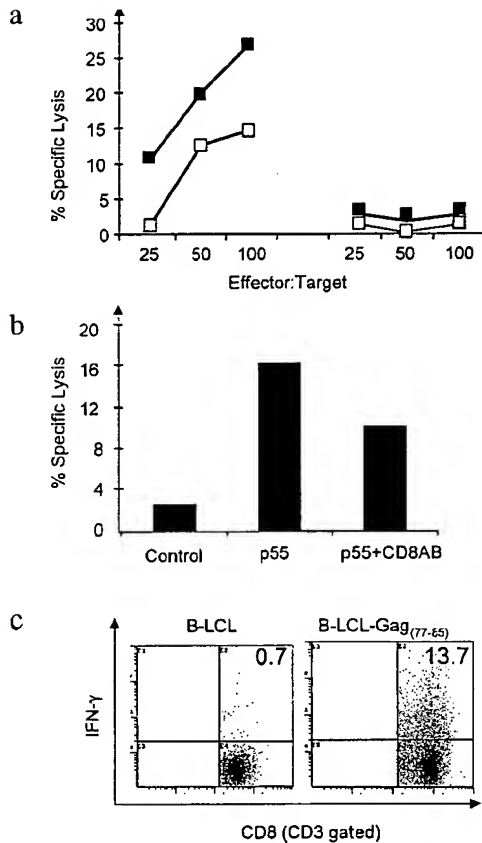
creased by introducing additional modifications consisting of one deletion and seven stop codons covering all three reading frames (Fig. 1a). Virions were derived from plasmid DNA encoding both the wild-type virus pLW and pLW/int by transfection of 293 cells. Supernatants were normalized for p24 contents and used to infect primary human cells susceptible to HIV-1 infection. As expected, the parental wild-type virus LW replicated in primary human lymphocytes, macrophages, and DC. In contrast, the integrase-mutant viral vector was unable to replicate in lymphocytes (Fig. 1b), macrophages (Fig. 1c), and DC (Fig. 1d), confirming that LW/int is a replication-defective viral vector.

DC are an attractive component of vaccines designed to induce T-cell responses in malignant and infectious diseases (26, 42) provided that the range of the presented epitopes and their immunostimulatory activity can be improved. They cap-
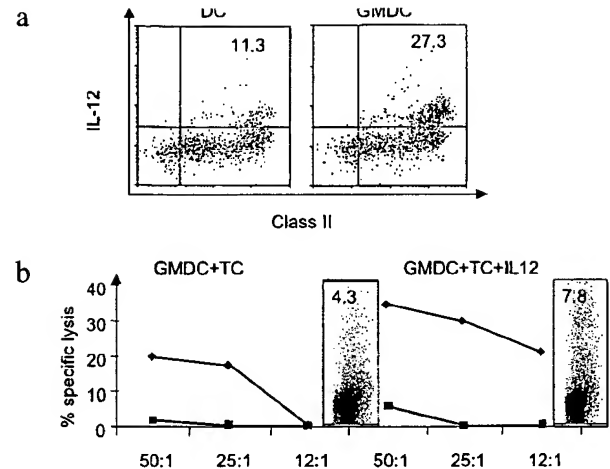
FIG. 5. Increased IL-12 secretion by GMDC activates T-cell priming and CTL response. (a) Increased IL-12 production by GMDC. DC (left panel) and GMDC (right panel) were stained with antibodies, analyzed by flow cytometry, and plotted as IL-12 (20C2; PharMingen) versus Class II (Immu-375; Immunotech). (b) IL-12 augments Th1-type primary immune responses. Naïve peripheral lymphocytes (TC) were primed with GMDC (1:10 ratio) in the presence (right) and absence (left) of 5 ng of IL-12 (R&D) per ml. Seven days later, HIV-1-specific CTL were tested against autologous target DC (17) pulsed with hen egg lysozyme (■) or with hi-HIV-1 (◆). The panels on the right of the graphs show the percentages of IFN-γ-producing CD8⁺ T cells after 3 days of priming with GMDC in the presence (right) and absence (left) of IL-12.

FIG. 4. Characterization of in vitro-induced HIV-specific CTL. (a) HIV-specific lysis. Naïve autologous lymphocytes were stimulated with GMDC (left) and control (right) autologous DCs. Seven days later, primed T cells were tested for HIV-specific CTL activity against autologous target macrophages (□) and macrophages pulsed with Gag (p55) protein (■). (b) Analysis of in vitro-primed HIV-1-specific CTL. Effector T cells induced by GMDC were tested against autologous target macrophages pulsed with the Gag protein (p55) (effector/target ratio, 50:1) in the presence and absence of CD8-specific antibodies (CD8AB). (c) GMDC activation of T cells specific to the dominant HLA-A*02-restricted Gag CTL epitope. GMDC derived from a naïve HLA-A*02 individual were used to prime autologous T cells. Seven days later the primed T cells were restimulated overnight with either autologous B-LCL cells (left) or B-LCL cells pulsed with HLA-A*02-restricted p17 Gag₇₇₋₈₅ (SLYNTVATL) peptide (right).

menting gene expression (32). Monocyte-derived DC (Fig. 2a) were transduced using plasmid DNA (pLW/int) complexed with PEI. Up to 50% of the resulting GMDC stained positively for Tat-specific antibodies by flow cytometry. Successful gene transfer was further demonstrated by the detection of HIV-1 p24 protein in the supernatant (Fig. 2b). Of course, the DC transduced by the wild-type HIV-1, pLW, produced larger amounts of p24 due to its virus replicative capacity.

We used flow-cytometric detection of intracellular IFN-γ to assess the magnitude of T-cell priming by GMDC. Control DC, GMDC, and DC pulsed with heat-inactivated HIV-1 (hi-HIV-1) were cocultured for 3 days with autologous naïve lymphocytes isolated from uninfected individuals. Results of a typical experiment are depicted in Fig. 2c: significant amounts of T cells (2.5% of CD3⁺ T lymphocytes, 1.2% of them CD8⁺

and 1.3% CD8⁻ T lymphocytes, representing mostly CD4⁺ cells) were primed by GMDC but not by either control DC or DC pulsed with hi-HIV-1. These results demonstrated that GMDC could efficiently prime naïve T cells within 4 days. Gene expression by DC seems to be essential for efficient T-cell priming, because pulsing with an extracellular antigen (hi-HIV-1) did not induce any significant T-cell priming.

GMDC-primed T cells were rechallenged with HIV-specific and control antigens presented by autologous B-LCL (Fig. 3). GMDC induced IFN-γ production by both CD8⁺ and CD4⁺ T cells independently of the sequence of the plasmid DNA used for their genetic modification, supporting previous findings demonstrating the high capacity of cultured DC for T-cell stimulation (26, 42). A total of 2.3% of CD8⁺ T cells and 3.2% of CD4⁺ T cells responded to HIV-specific restimulation after priming with LW/int-transduced GMDC, compared to 0.8 and 2.2%, respectively, after pGFP-transduced-GMDC priming. Moreover, restimulation of HIV-specific T cells with a control antigen resulted only in nonspecific IFN-γ production. These experiments demonstrated that GMDC can convert naïve lymphocytes to both CD8⁺ and CD4⁺ HIV-specific T cells.

The next question was whether these activated lymphocytes had acquired the ability to kill cells presenting HIV-1 antigens. Since macrophages are one of the important reservoirs of HIV-1, these cells were used as a target for measuring CTL activity. T cells incubated with control DC did not kill HIV-1 Gag (p55)-loaded macrophages. In contrast, GMDC-primed T cells exerted a vigorous CTL activity (Fig. 4a). To identify the cells that killed Gag (p55)-pulsed macrophages, the CTL assay was performed in the presence and absence of CD8-specific

a

mRNA

Interdigitating DC
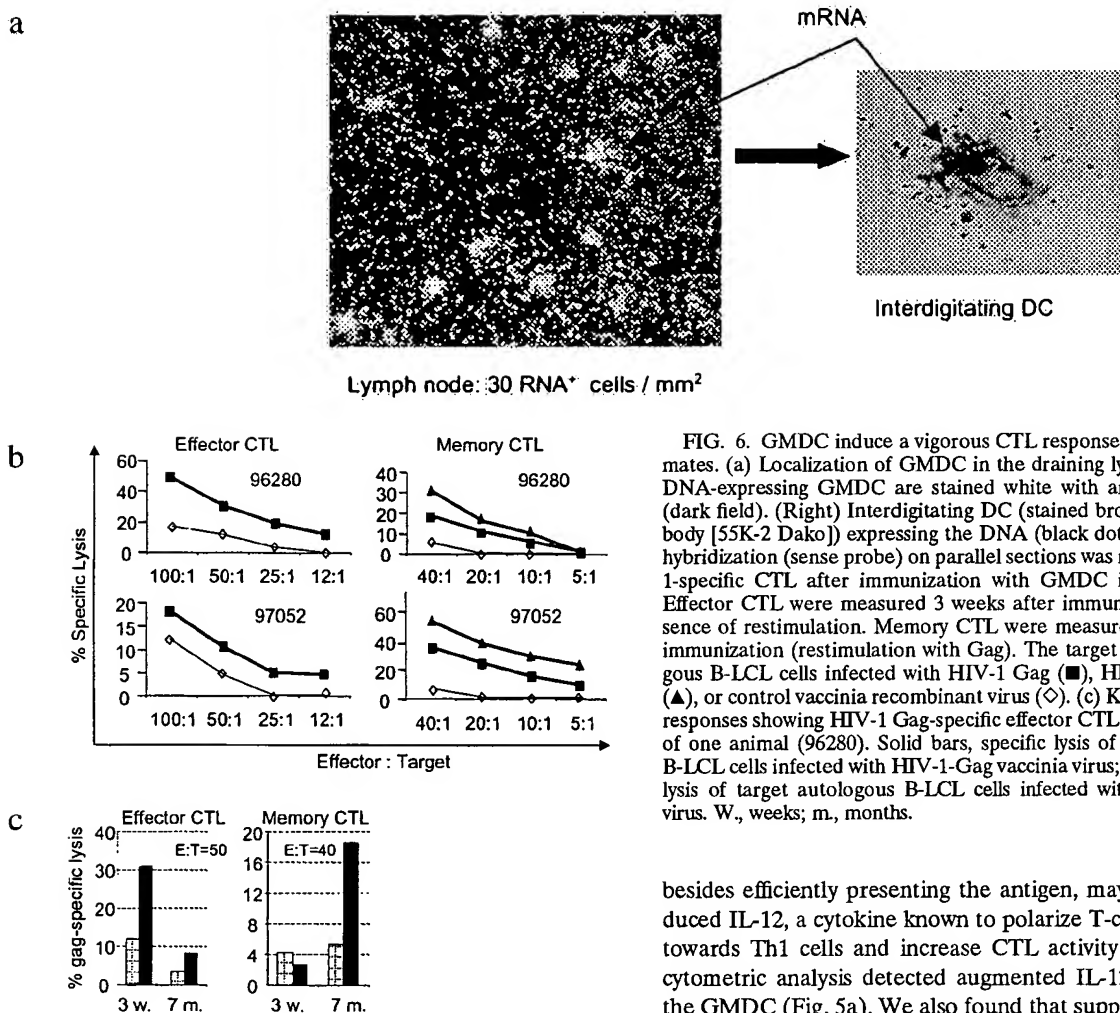
Lymph node: 30 RNA⁺ cells / mm²

b

c

FIG. 6. GMDC induce a vigorous CTL response in nonhuman primates. (a) Localization of GMDC in the draining lymph node. (Left) DNA-expressing GMDC are stained white with an antisense probe (dark field). (Right) Interdigitating DC (stained brown with p55 antibody [55K-2 Dako]) expressing the DNA (black dots). Control in situ hybridization (sense probe) on parallel sections was negative. (b) HIV-1-specific CTL after immunization with GMDC in two macaques. Effector CTL were measured 3 weeks after immunization in the absence of restimulation. Memory CTL were measured 7 months after immunization (restimulation with Gag). The target cells were autologous B-LCL cells infected with HIV-1 Gag (■), HIV-1 Gag-Pol-Env (▲), or control vaccinia recombinant virus (◇). (c) Kinetics of the CTL responses showing HIV-1 Gag-specific effector CTL and memory CTL of one animal (96280). Solid bars, specific lysis of target autologous B-LCL cells infected with HIV-1-Gag vaccinia virus; open bars specific lysis of target autologous B-LCL cells infected with control vaccinia virus. W., weeks; m., months.

antibodies (Fig. 4b). These antibodies partially inhibited HIV-specific cytotoxicity, suggesting the participation of CD8⁺ T cells in the lysis. These results confirmed that GMDC can activate CD8⁺ T cells and demonstrated that these cells acquired HIV-specific cytotoxic activity.

We also studied a naïve HLA-A*02 type donor to further characterize the GMDC-primed CD8⁺ T cells (45). Seven days after GMDC priming, T cells were restimulated either with autologous B-LCL cells or with B-LCL cells pulsed with the dominant Gag peptide. Of the GMDC-primed CD8⁺ T cells, 14% responded by IFN-γ production to the dominant Gag epitope, whereas only 1% responded to the control stimulation (Fig. 4c). These results confirmed the specificity and the magnitude of the HIV-1-specific CD8⁺ T cell response induced by the GMDC. Similar high frequencies of HLA-A*02-restricted Gag epitope-specific circulating CD8⁺ T cells have been detected in patients during acute HIV-1 infection (46), suggesting that GMDC are capable of priming a similar T-cell population to the level elicited by live HIV-1.

We questioned why the GMDC primed so many T cells in such a short period of time. We postulated that the GMDC,

besides efficiently presenting the antigen, may have also produced IL-12, a cytokine known to polarize T-cell development towards Th1 cells and increase CTL activity (11, 16). Flow-cytometric analysis detected augmented IL-12 production by the GMDC (Fig. 5a). We also found that supplementary IL-12 in the culture media enhanced T-cell priming by GMDC and doubled the number of IFN-γ-producing CD8⁺ T cells (Fig. 5b). Analysis of the induction of CTL activity by GMDC also confirmed the T-cell-priming results. Increased IL-12 concentration substantially augmented the HIV-1-specific CTL activity but not the unspecific CTL activity (Fig. 5b). These results suggest an IL-12-mediated mechanism to explain both the potency and the antigen specificity of the GMDC-induced functional T cells.

Antigen-presenting DC can transport antigen efficiently from the periphery to the lymphoid tissue. To study the fate of GMDC in vivo, autologous GMDC were injected subcutaneously into the thigh of a rhesus macaque. One day later, the draining lymph node was removed and gene expression was detected by in situ hybridization. Exceptionally high numbers of DNA expressing GMDC were found in the lymph node. Some of these cells had already interdigitated into the T-cell area and stained positively with an antibody (p55) specific for lymph node DC (43) (Fig. 6a). Quantitative analysis of DNA expressing DC in the draining lymph node revealed ca. 30 cells/mm². A conservative estimation of the total positive cells per lymph node resulted in ca. 60,000 cells. In striking contrast, only 50 to 100 gene-expressing DC were found in the lymph

node after needle injection of DNA in the muscle or the skin or after DNA-containing particle bombardment into the skin with a gene gun (33). These results suggest that GMDC can migrate efficiently to the T-cell areas of the draining lymph node (43) and express plasmid DNA.

HIV-1 reproducibly infects pigtailed macaques (*Macaca nemestrina*) (1, 34); therefore, GMDC were generated from monocytes of two pigtailed macaques using the same procedure as described for the human DC, to confirm our in vitro results. Autologous GMDC were injected into each of two animals; no side effects, such as obvious discomfort or fever, were observed after injection. Both animals maintained normal blood chemistry, complete blood cell counts, and $CD4^+$-lymphocyte numbers immediately after inoculation and throughout the experimental follow-up of 7 months. Several attempts to isolate HIV-1 failed, as would be expected from inoculation by a replication-defective viral vector. None of the animals seroconverted, confirming the absence of replication-competent HIV-1 and revealing the absence of humoral immune responses. However, we expected rapid activation of naïve T cells and the development of HIV-specific CTL in the animals because we had seen potent T-cell responses induced by GMDC in vitro and a high rate of DNA expression in the lymph nodes. Therefore, we assayed the cytotoxic activity of PBMC after immunization without antigenic restimulation, thus limiting our analyses to effector CTL (12). Both animals developed effector CTL within 3 weeks (Fig. 6b, left panels), suggesting that efficient T-cell priming had been obtained in vivo. Seven months later, potent HIV-1-specific CTL responses were detected in both animals using a conventional assay detecting mainly memory CTL after in vitro antigenic (Gag) restimulation of T cells (Fig. 6b, right panels). Longitudinal examination of the CTL data demonstrated an HIV-1-specific T-cell activity expected from a very efficient T-cell priming (Fig. 6c): early after immunization, most of the T cells were freshly activated; therefore, they could lyse target cells in the absence of in vitro restimulation. This is characteristic of effector T cells. The activity of HIV-specific effector T cells decreased within 7 months, suggesting the possible elimination of antigen presented by GMDC. Memory CTL representing T cells are capable of proliferation and subsequent cytotoxicity after antigenic stimulation. Seven months after GMDC immunization potent HIV-specific CTL responses were detected after in vitro antigenic restimulation, suggesting the establishment of a long-term memory CTL response. These results confirmed our in vitro data and demonstrated in vivo the development of a potent, long-lasting, HIV-1-specific, T-cell-polarized immune response induced by GMDC.

DISCUSSION

We have described here a new technology to generate potent antigen-presenting DC for the stimulation of broad and long-lasting HIV-specific T-cell immunity. This novel approach utilizes in vivo production of HIV-1 antigens from a circular plasmid DNA that encodes a replication- and integration-defective lentivirus vector. While lentiviruses integrate permanently into the host's genome, plasmid DNA does not. Instead, they are progressively lost during cell division. This renders the plasmid DNA encoding the integrase-defective lentivirus vec-

tor safer than the integration-competent retrovirus vectors used for experimental human gene therapy. The use of a plasmid DNA also circumvented the gene expression problem, because sequences inserted between the two long terminal repeats reduced the interference between the promoters and allowed the genes to be expressed efficiently (5). This strategy of transient and efficient antigen expression thus combines improved safety and efficacy features.

Genetic modification of DC by PEI-DNA complexes offers additional advantages. The DNA used in the present examples encodes nearly all the proteins of HIV-1, and there is no indication that this DNA has approached the size limit for gene transfer. The designer of future vaccines is not likely to be limited to the use of a single peptide or protein if this technique is used. This suggests that it may be feasible to construct a single vaccine that would be effective for individuals infected with different clades of HIV-1. Moreover, autologous GMDC process the DNA-encoded proteins in vivo for major histocompatibility complex class I-and class II-specific peptide presentation to the T cells. Therefore, GMDC technology does not require HLA typing of patients, in contrast to methods using HLA-matched peptides for DC priming. Clinically, ex vivo culture of DC has been optimized and used in experimental cancer therapies. Transduction of DC with PEI is simple, because any plasmid DNA can be used without the need of cumbersome cloning techniques. The plasmid DNA required to generate GMDC (0.001 mg per 100,000 DC) and the PEI can also be manufactured in high scale. In addition, no undesired immune response is expected to be generated against PEI, as occurs with viral vectors (e.g., adenovirus vectors [10, 47]). Finally, GMDC are likely to be eliminated from the body as soon as the antigen-specific CTL develops, since antigen-presenting DC are good targets for CTL.

This study presents the first experimental evidence that the cellular arm of the immune system can be deliberately activated independently of the humoral arm. This restricted response is reminiscent of the immune status of high-risk uninfected sex workers, who had HIV-1-specific cellular immune responses but no HIV-1 antibody responses (37–39). It is plausible that the antigen presentation through intracellular DNA expression by GMDC, as opposed to the uptake of extracellular antigen by DC, has polarized the immune response toward its cellular arm. Endogenous synthesis of foreign protein antigens is known to elicit class I-restricted $CD8^+$ CTL responses. GMDC in this experiment were found to express significant amounts of both HIV-1 protein and IL-12, a cytokine known to polarize T cells towards Th1 responses (7, 16, 25). The location and the unprecedentedly high rate of DNA expression by GMDC in the lymphoid organs have also played a major role in the efficiency of antigen presentation. GMDC activated both CTL and T-helper responses that contributed to the generation of potent and long-lasting HIV-specific T cells in vivo in nonhuman primates.

The hope that T-cell-mediated immune control can be achieved after early treatment of acute HIV-1 infection has been raised (21, 23, 36). The question is now whether a therapeutic vaccine can be given to HIV-1-infected patients, which will boost their T-cell immunity and perhaps restore their immune system enough to allow some safe periods off antiretroviral drugs. Although the ability of HIV-specific cellular im-

munity to permanently control virus replication has not yet been proven, it could be tested by combining highly active antiretroviral therapies with the genetic immunization approach described here.

REFERENCES

1. Agy, M. B., A. Schmidt, M. J. Florey, B. J. Kennedy, G. Schaefer, M. G. Katze, L. Corey, W. R. Morton, and M. L. Bosch. 1997. Serial in vivo passage of HIV-1 infection in *Macaca nemestrina*. Virology 238:336–343.
2. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. Nature 392:245–252.
3. Bhardwaj, N., A. Bender, N. Gonzalez, L. K. Bui, M. C. Garrett, and R. M. Steinman. 1995. Stimulation of human anti-viral CD8+ cytolytic T lymphocytes by dendritic cells. Adv. Exp. Med. Biol. 378:375–379.
4. Boussif, O., F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, and J. P. Behr. 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. USA 92:7297–7301.
5. Cara, A., A. Cereseto, F. Lori, and M. S. Reitz, Jr. 1996. HIV-1 protein expression from synthetic circles of DNA mimicking the extrachromosomal forms of viral DNA. J. Biol. Chem. 271:5393–5397.
6. Cara, A., F. Guarnaccia, M. Reitz, R. Gallo, and F. Lori. 1995. Self-limiting, cell-type dependent replication of an integrase defective HIV-1 in human macrophages but not in human T-lymphocytes. Virology 208:646–650.
7. Cella, M., M. Salio, Y. Sakakibara, H. Langen, I. Julkunen, and A. Lanzavecchia. 1999. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. J. Exp. Med. 189:821–829.
8. Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science 258:1938–1941.
9. Evans, D. T., D. H. O'Connor, P. Jing, J. L. Dzuris, J. Sidney, J. da Silva, T. M. Allen, H. Horton, J. E. Venham, R. A. Rudersdorf, T. Vogel, C. D. Pauza, R. E. Bontrop, R. DeMars, A. Sette, A. L. Hughes, and D. I. Watkins. 1999. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus env and Nef. Nat. Med. 5:1270–1276.
10. Granelli-Piperno, A., V. Finkel, E. Delgado, and R. M. Steinman. 1999. Virus replication begins in dendritic cells during the transmission of HIV-1 from mature dendritic cells to T cells. Curr. Biol. 9:21–29.
11. Heufler, C., F. Koch, U. Stanzl, G. Topar, M. Wysocka, G. Trinchieri, A. Enk, R. M. Steinman, and G. Schuler. 1996. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. Eur. J. Immunol. 26:659–668.
12. Jacob, J., and D. Baltimore. 1999. Modelling T-cell memory by genetic marking of memory T cells in vivo. Nature 399:593–597.
13. Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho. 1999. Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiency virus-infected macaques. J. Exp. Med. 189:991–998.
14. Kalams, S. A., P. J. Goulder, A. K. Shea, N. G. Jones, A. K. Trocha, G. S. Ogg, and B. D. Walker. 1999. Levels of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte effector and memory responses decline after suppression of viremia with highly active antiretroviral therapy. J. Virol. 73:6721–6728.
15. Kent, S. J., A. Zhao, S. J. Best, J. D. Chandler, D. B. Boyle, and I. A. Ramshaw. 1998. Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. J. Virol. 72:10180–10188.
16. Kim, J. J., V. Ayyavoo, M. L. Bagarazzi, M. A. Chattergoon, K. Dang, B. Wang, J. D. Boyer, and D. B. Weiner. 1997. In vivo engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen. J. Immunol. 158:816–826.
17. Knight, S. C., B. A. Askonas, and S. E. Macatonia. 1997. Dendritic cells as targets for cytotoxic T lymphocytes. Adv. Exp. Med. Biol. 417:389–394.
18. Knight, S. C., S. Iqball, M. S. Roberts, S. Macatonia, and P. A. Bedford.

19. Kundig, T. M., M. F. Bachmann, C. DiPaolo, J. J. Simard, M. Battegay, H. Lother, A. Gessner, K. Kuhlcke, P. S. Ohashi, H. Hengartner, R. Zinkernagel, et al. 1995. Fibroblasts as efficient antigen-presenting cells in lymphoid organs. Science 268:1343–1347.
20. Labat, M. F., A. M. Steffan, C. Brisson, H. Perron, O. Feugeas, P. Furstenberger, F. Oberling, E. Brambilla, and J. P. Behr. 1996. An electron microscopy study into the mechanism of gene transfer with lipopolyamines. Gene Ther. 3:1010–1017.
21. Lisziewicz, J., E. Rosenberg, J. Lieberman, H. Jessen, L. Lopalco, R. Siliciano, B. Walker, and F. Lori. 1999. Control of HIV despite the discontinuation of antiretroviral therapy. N. Engl. J. Med. 340:1683–1684.
22. Lori, F., L. Hall, P. Lusso, M. Popovic, P. Markham, G. Franchini, and M. S. Reitz, Jr. 1992. Effect of reciprocal complementation of two defective human immunodeficiency virus type 1 (HIV-1) molecular clones on HIV-1 cell tropism and virulence. J. Virol. 66:5553–5560.
23. Lori, F., M. G. Lewis, J. Xu, G. Varga, D. J. Zinn, C. Crabbs, W. Wagner, J. Greenhouse, P. Silvera, J. Yalley-Ogunro, C. Tinelli, and J. Lisziewicz. 2000. Control of SIV rebound through structured treatment interruptions during early infection. Science 290:1591–1593.
24. Ludewig, B., S. Ehl, U. Karrer, B. Odermatt, H. Hengartner, and R. M. Zinkernagel. 1998. Dendritic cells efficiently induce protective antiviral immunity. J. Virol. 72:3812–3818.
25. Macatonia, S. E., N. A. Hosken, M. Litton, P. Vieira, C. S. Hsieh, J. A. Culpepper, M. Wysocka, G. Trinchieri, K. M. Murphy, and A. O'Garra. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T-cells. J. Immunol. 154:5071–5079.
26. Mellman, I., S. J. Turley, and R. M. Steinman. 1998. Antigen processing for amateurs and professionals. Trends Cell Biol. 8:231–237.
27. Morgan, R. A., and R. Walker. 1996. Gene therapy for AIDS using retroviral mediated gene transfer to deliver HIV-1 antisense TAR and transdominant Rev protein genes to syngeneic lymphocytes in HIV-1 infected identical twins. Hum. Gene Ther. 7:1281–1306.
28. Naldini, L., U. Blomer, F. H. Gage, D. Trono, and I. M. Verma. 1996. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc. Natl. Acad. Sci. USA 93:11382–11388.
29. Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D. D. Ho, D. F. Nixon, and A. J. McMichael. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 279:2103–2106.
30. Ogg, G. S., X. Jin, S. Bonhoeffer, P. Moss, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, A. Hurley, M. Markowitz, D. D. Ho, A. J. McMichael, and D. F. Nixon. 1999. Decay kinetics of human immunodeficiency virus-specific effector cytotoxic T lymphocytes after combination antiretroviral therapy. J. Virol. 73:797–800.
31. Pitcher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino, and L. J. Picker. 1999. HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. Nat. Med. 5:518–525.
32. Pollard, H., J. S. Remy, G. Loussouarn, S. Demolombe, J. P. Behr, and D. Escande. 1998. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J. Biol. Chem. 273:7507–7511.
33. Porgador, A., K. R. Irvine, A. Iwasaki, B. H. Barber, N. P. Restifo, and R. N. Germain. 1998. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. J. Exp. Med. 188:1075–1082.
34. Prince, A. M., and L. Andrus. 1998. AIDS vaccine trials in chimpanzees. Science 282:2195–2196.
35. Riddell, S. R., M. Elliott, D. A. Lewinsohn, M. J. Gilbert, L. Wilson, S. A. Manley, S. D. Lupton, R. W. Overell, T. C. Reynolds, L. Corey, and P. D. Greenberg. 1996. T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. Nat. Med. 2:216–223.
36. Rosenberg, E., M. Altfeld, S. H. Poon, M. N. Phillips, B. M. Wilkes, R. L. Eldridge, G. K. Robbins, R. T. D'Aquilla, P. J. R. Goulder, and B. D. Walker. 2000. Immune control of HIV-1 after early treatment of acute infection. Nature 407:523–526.
37. Rowland-Jones, S., T. Dong, P. Krausa, J. Sutton, H. Newell, K. Ariyoshi, F. Gotch, S. Sabally, T. Corrah, J. Kimani, K. MacDonald, F. Plummer, J. Ndinya-Achola, H. Whittle, and A. McMichael. 1998. The role of cytotoxic T-cells in HIV infection. Dev. Biol. Stand. 92:209–214.
38. Rowland-Jones, S., J. Sutton, K. Ariyoshi, T. Dong, F. Gotch, S. McAdam, D. Whitby, S. Sabally, A. Gallimore, T. Corrah, et al. 1995. HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. Nat. Med. 1:59–64.
39. Rowland-Jones, S. L., T. Dong, K. R. Fowke, J. Kimani, P. Krausa, H. Newell, T. Blanchard, K. Ariyoshi, J. Oyugi, E. Ngugi, J. Bwayo, K. S. MacDonald, A. J. McMichael, and F. A. Plummer. 1998. Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. J. Clin. Invest. 102:1758–1765.

40. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J. Exp. Med. 179:1109–1118.

41. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8$^+$ lymphocytes. Science 283:857–860.

42. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. 9:271–296.

43. Steinman, R. M., M. Pack, and K. Inaba. 1997. Dendritic cells in the T-cell areas of lymphoid organs. Immunol. Rev. 156:25–37.

44. Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica. 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. 9:1551–1560.

45. Tsomides, T. J., B. D. Walker, and H. N. Eisen. 1991. An optimal viral peptide recognized by CD8$^+$ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein. Proc. Natl. Acad. Sci. USA 88:11276–11280.

46. Wilson, J. D., G. S. Ogg, R. L. Allen, C. Davis, S. Shaunak, J. Downie, W. Dyer, C. Workman, S. Sullivan, A. J. McMichael, and S. L. Rowland-Jones. 2000. Direct visualization of HIV-1 specific cytotoxic T lymphocytes during primary infection. AIDS 14:225–233.

47. Zhong, L., A. Granelli-Piperno, Y. Choi, and R. M. Steinman. 1999. Recombinant adenovirus is an efficient and non-perturbing genetic vector for human dendritic cells. Eur. J. Immunol. 29:964–972.

48. Zinkernagel, R. M., S. Ehl, P. Aichele, S. Oehen, T. Kundig, and H. Hengartner. 1997. Antigen localisation regulates immune responses in a dose- and time-dependent fashion: a geographical view of immune reactivity. Immunol. Rev. 156:199–209.